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Abstract

The objective of this proposal is to determine the effectiveness of tumor stromal targeting using radiolabeled antibodies that deliver cytotoxic payloads to breast cancer stromal fibroblasts expressing fibroblast activation protein (FAP). The central hypothesis to be tested is that targeting breast cancer stroma will result in enhanced tumor cytotoxicity compared to targeting the breast cancer cells themselves. FAP is a fibroblast cell surface glycoprotein that is selectively expressed by tumor stromal fibroblasts in breast tumors, but is not significantly expressed by breast cancer cells, normal fibroblasts, or other normal tissues. We have identified an appropriate animal model that allows for evaluation of both stromal and epithelial targeting of BT-474 xenografts. Although epithelial targeting was accomplished, stromal targeting of FAP remains under development utilizing additional antibody reagents. These initial biodistribution studies will inform future therapeutic studies to investigate a radioimmunotherapeutic strategy for preclinical treatment of breast cancers in an animal model. If a radioimmunotherapy strategy is effective in inhibiting breast cancer growth in an animal model, it may lead to a novel therapeutic approach targeting the tumor stroma in patients with breast cancer.

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INTRODUCTION

Clinical effectiveness of radioimmunotherapy (RIT) in lymphomas has led to the FDA approval of the radiolabeled antibodies Ibritumomab Tiuxetan and Tositumomab. Some of the major impediments for successful use of RIT in patients with breast cancer include: 1) insufficient selectivity of the tumor antigen leading to host toxicities, and 2) poor tumor penetration of large antibody molecules due to the impaired vascular access and high interstitial pressure of tumors. These obstacles may be overcome if an exquisitely tumor-selective antigen in the tumor stroma is targeted with antibody fragments that more easily penetrate the tumor mass. Fibroblast activation protein (FAP) may be such a target. FAP is a fibroblast cell surface glycoprotein that is selectively expressed by tumor stromal fibroblasts in breast tumors, but is not significantly expressed by breast cancer cells, normal fibroblasts, or other normal tissues [1]. We have shown that FAP potentiates tumor growth in vivo [2].

The objective of this proposal is to determine the effectiveness of tumor stromal targeting using radiolabeled antibodies that deliver cytotoxic payloads to breast cancer stromal fibroblasts expressing FAP. **The *central hypothesis* to be tested is that targeting breast cancer stroma will result in enhanced tumor cytotoxicity compared to targeting the breast cancer cells themselves.** Targeting the tumor stroma may be advantageous due to the close physical proximity of the tumor stromal cells to the tumor capillaries, allowing easier accessibility of circulating antibodies in the blood to their targets. Since stromal cells have fewer genetic alterations compared to transformed malignant cells, reactive stromal cells may express target antigens in a more uniform and stable fashion compared to malignant cells.

BODY

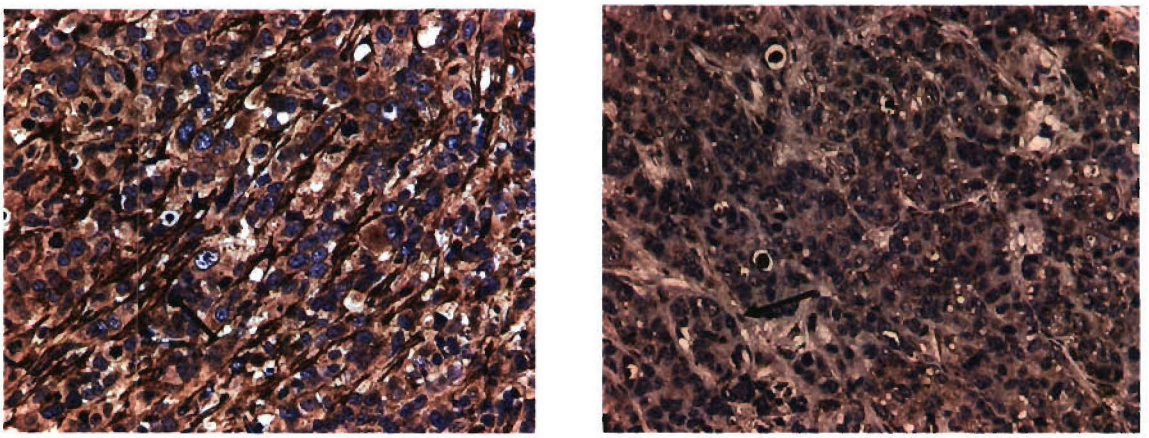
The primary objective of this proposal is to characterize the effectiveness of radioimmunotherapy employing antibodies that target stromal FAP in a breast cancer xenograft model. This proposal aims to test the hypothesis that radiolabeled diabodies targeting FAP in the breast cancer stroma will have enhanced therapeutic effectiveness compared to radiolabeled antibodies targeting HER2 on the breast cancer cells.

Identification of Breast Xenografts with FAP Induction

A number of breast cancer xenografts were stained for stromal fibroblast induction of FAP. These breast cancer cell lines included BT474, MCF7, MD231, and MD361. BT474 had the most robust induction of stromal FAP as seen in **Figure 1** below. Paraffin embedded tumor specimens were deparaffinized to distilled water using xylene and alcohol. Microwave antigen retrieval was performed in 10 mM Citrate Buffer ph 6.0 for 10 minutes. After peroxidase and protein blocking, the primary anti-FAP rabbit antibody was applied in a dilution of 1:1200 overnight, and the secondary goat anti-rabbit incubated for 30 minutes. Avidin-biotin complex streptavidin-horseradish peroxidase was applied prior to the addition of chromogen-DAB substrate. Specimens were counterstained with Hematoxylin and dehydrated to xylene. The arrow in **Figure 1, Left Panel** demonstrates intense stromal staining of FAP in BT474 xenografts, as contrasted by the lack of stromal

staining (arrow) in the MD361 xenograft (**Figure 1, Right Panel**). Thus, BT-474 breast cancer xenografts induce robust stromal FAP expression, and were utilized for radioimmunotherapeutic evaluations.

Figure 1. Immunohistochemical staining of FAP in breast cancer xenografts

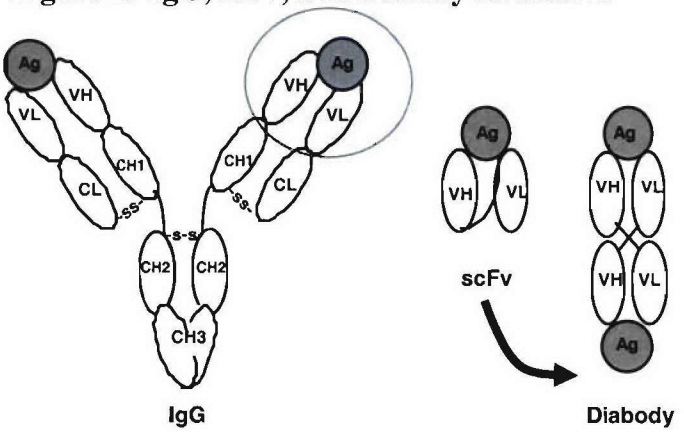


FAP (+) BT474 Xenografts **FAP (-) MDA-MB-361 Xenografts**

Anti-FAP antibodies

Single-chain antibodies (scFv) were identified using phage display techniques by panning a naïve human phage display library containing 10^{10} unique phage [3] against recombinant murine FAP. The scFv antibody E8 was identified as an antibody targeting murine FAP and converted into a diabody format for its favorable pharmacokinetic properties and prolonged in vivo half-life. As shown in **Figure 2,** the variable heavy (VH) and light (VL) domains of an IgG molecule are joined by a 15 amino acid spacer to form a single chain Fv (scFv) molecule capable of binding a single antigen (Ag). Shortening the spacer to 5 amino acids prevents association between the light and heavy chains of a single scFv molecule. Their high affinity for each other favors the formation of noncovalent dimers (diabodies).

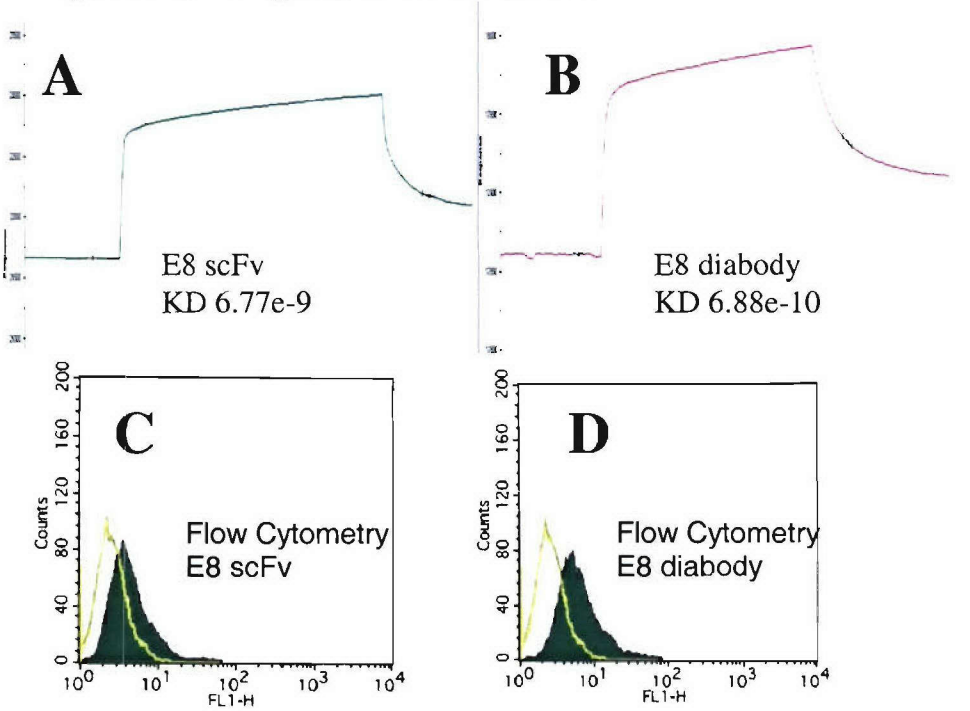
Figure 2. IgG, scFv, and diabody structures



These antibodies were characterized by BIAcore and FACS analysis [4]. Surface plasmon resonance (SPR) was performed using BIAcore technology. The SPR response reflects the change in the mass concentration as molecules bind to or dissociate from a ligand, immobilized on a sensor chip. ScFv association/dissociation rates and affinities of the E8 scFvs and diabodies for FAP was determined by passing serially diluted samples of the scFv over a FAP-ECD immobilized CM5 chip. The sensograms in **Figure 3** show the interaction of the scFv E8 (**Figure 3, Panel A**) and E8 diabody (**Figure 3, Panel B**) with the immobilized FAP-ECD, demonstrating a K_d of 6.77 x 10^{-9} M and 6.88 x 10^{-10}M for E8 scFv and diabody, respectively.

Flow cytometry was performed using HEK293 cells stably transfected with FAP. HEK293-FAP cells were incubated with the scFv primary antibody on ice, followed by a secondary mouse anti-His antibody directed against the His epitope tag of the scFv, followed by FITC-conjugated goat anti-mouse tertiary antibody, and fixed with 1% paraformaldehyde in PBS. HEK293-FAP cells incubated only with the secondary and tertiary antibodies were used as a negative control. The samples were analyzed on a BD FACScan Instrument as shown in **Figure 3**. The scFv E8 (**Figure 3, Panel C**) and E8 diabody (**Figure 3, Panel D**) demonstrates FAP specific binding as seen by the right shift of the closed curve of the E8 FAP antibodies compared to the open negative control curve of non-specific His antibody. The E8 antibodies did not bind to the negative control cells of HEK293 transfected with vector only (mock) controls (data not shown).

Figure 3. Binding of E8 antibodies to FAP

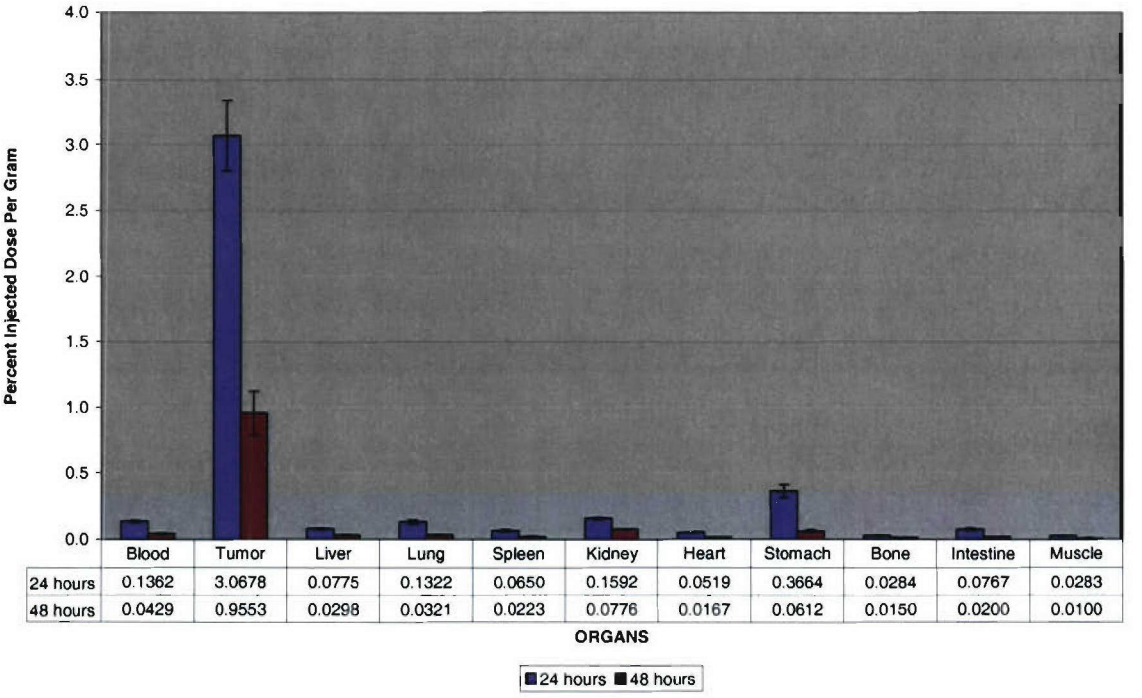


Effective targeting of BT474 xenografts with Anti-HER2 antibody

BT-474 breast cancer cells overexpress HER2, and maintain this HER2 overexpression when xenografted onto immunodeficient mice (data not shown). Biodistribution studies of ^{125}I-labeled antibodies targeting HER2 were performed to inform therapeutic studies.

Biodistribution analyses were performed using ALM (anti-HER2) antibody radiolabeled with ^{125}I using Iodogen. Cohorts of 5 scid mice bearing BT-474 xenografted tumors received 20 μCi per mouse of radioiodinated ALM via tail vein injection. Percent injected dose per gram of tissue (%ID/g) and cumulative tumor: organ ratios was determined by necropsy at 24 and 48 hours post injection as previously described [5]. As shown in **Figure 4** below, effective targeting of the anti-HER2 antibody was seen in vivo of BT-474 xenografts with tumor: organ ratios of > 20:1.

Figure 4. Biodistribution of I125-ALM in BT-474 containing scid mice



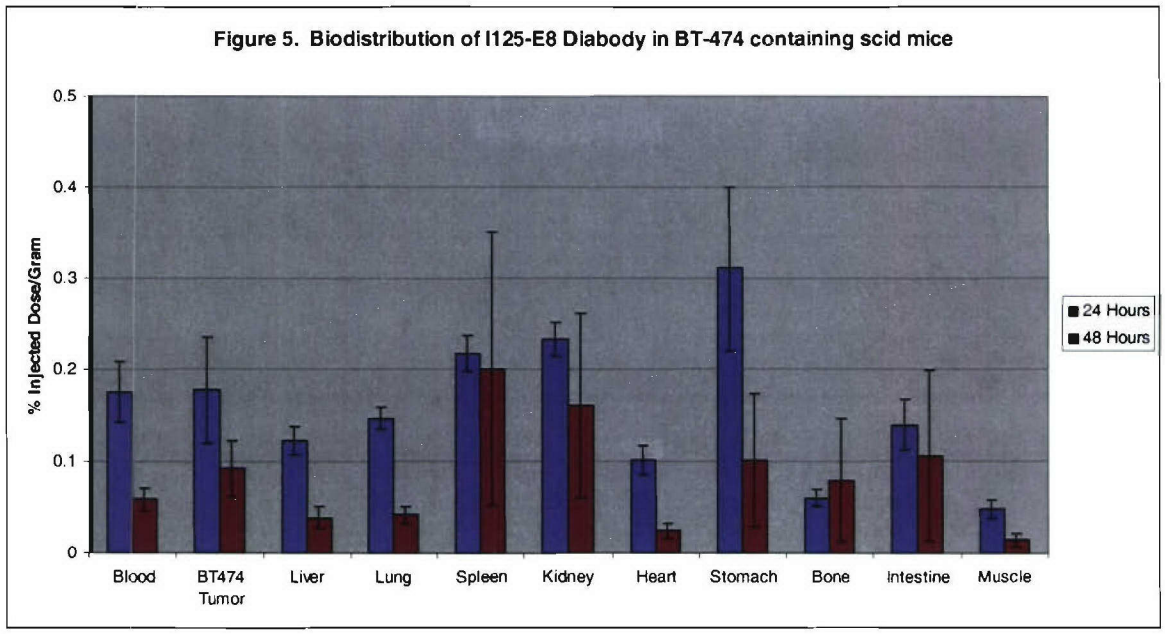
	Blood	Tumor	Liver	Lung	Spleen	Kidney	Heart	Stomach	Bone	Intestine	Muscle
24 hours	0.1362	3.0678	0.0775	0.1322	0.0650	0.1592	0.0519	0.3664	0.0284	0.0767	0.0283
48 hours	0.0429	0.9553	0.0298	0.0321	0.0223	0.0776	0.0167	0.0612	0.0150	0.0200	0.0100

Initial unsuccessful targeting of BT-474 xenografts with anti-FAP antibodies

BT-474 breast cancer cells when xenografted onto nude mice induce stromal FAP expression on the stromal fibroblasts as shown in **Figure 1, Left Panel**. Biodistribution studies of ^{125}I-labeled E8 diabodies targeting FAP were performed to inform therapeutic studies using the methodology described above for the anti-HER2 antibody ALM. As shown in Figure 5 below, E8 diabodies did not target stromal FAP in vivo, with a tumor: organ ratio of approximately 1.

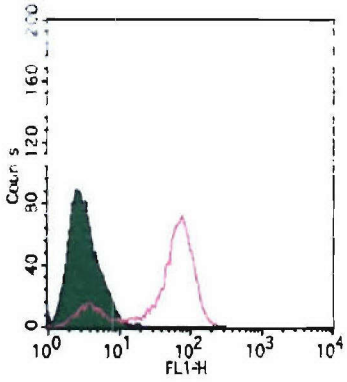
There are a number of possibilities to explain the lack of E8 diabody targeting of FAP. These include:

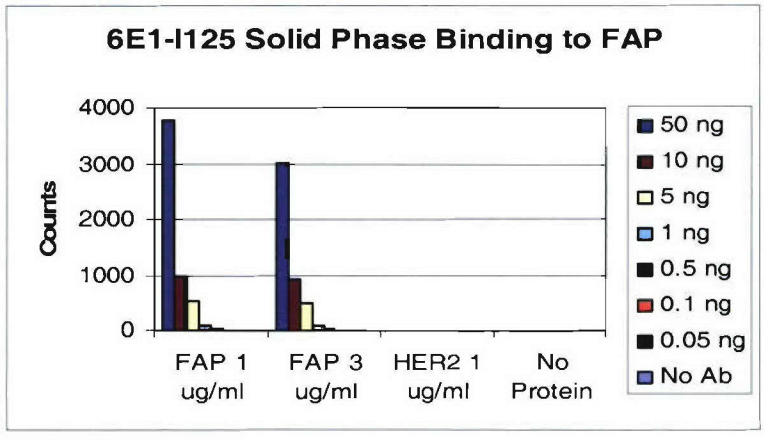
1. Disruption of the E8 binding to FAP through the I125 labeling process
2. Degradation of the radiolabeled E8 antibody in vivo
3. Poor penetration of the E8 diabody into the tumor mass
4. Inadequate binding of E8 diabody to in vivo FAP
5. Inadequate FAP expression on the stromal fibroblasts



To address these concerns, we have initiated a biodistribution study of a IgG monoclonal antibody that targets murine FAP, using both BT-474 and HEK-FAP xenografts. Given the relative stability of IgGs in the labeling process and the predictable in vivo pharmacokinetics, this experiment will provide the proof-of-principle of the ability of antibodies to target murine FAP in vivo. The positive control of HEK-FAP xenografts with its abundant FAP expression on the cell surface will provide optimal in vivo conditions for murine FAP targeting. As shown in **Figure 6, Left Panel**, the rabbit monoclonal antibody 6E1 effectively binds to HEK-FAP cells as analyzed by flow cytometry. Live cell binding assay of ^{125}I labeled 6E1 antibody demonstrates 30% binding to HEK-FAP cells (data not shown), and solid phase assessment of radiolabeled 6E1 given in decreasing concentrations shows effective targeting of FAP (**Figure 6**).

Figure 6. Binding of rabbit monoclonal 6E1 to murine FAP





Biodistribution studies using 6E1 to target stromal FAP in BT-474 xenografts, and HEK-FAP xenografts have been initiated and are nearing completion. This will inform the utility of anti-FAP antibodies to targeting tumor xenografts in vivo.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of BT474 breast cancer xenografts that induce strong stromal expression of FAP.
- Identification of scFv antibodies that bind with K_d 6.77e-9 affinity to murine FAP. This E8 antibody also binds to FAP in its native conformation on HEK293-FAP cells.
- Synthesis of anti-FAP E8 diabodies that bind with 6.88e-10 K_d affinity to murine FAP. This E8 diabody also binds FAP in its native conformation on HEK293-FAP cells.
- Effective targeting in vivo of anti-HER2 antibody ALM to BT-474 xenografts, which overexpress HER2.
- Lack of effective targeting of E8 diabodies to BT-474 xenografts that induce stromal FAP expression. This preliminary result is being reassessed with additional experimentation.
- Identification of rabbit monoclonal antibody 6E1 that retains its affinity for murine FAP after radiolabeling. This antibody is being used for additional biodistribution studies targeting murine FAP.

REPORTABLE OUTCOMES

- Cheng JD, Valianou M, Canutescu AA, Jaffe EK, Lee HO, Wang H, Lai JH, Bachovchin WW, Weiner LM. Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth. Mol Cancer Ther. 2005 Mar;4(3):351-60. (describes 6E1 antibody)
- AACR abstract of above accomplishments in preparation

CONCLUSION

Human breast cancers induce expression of FAP in the surrounding stromal fibroblasts to potentiate tumor growth and invasion. This research project proposes using engineered antibody fragments to deliver a cytotoxic payload to these tumor stromal fibroblasts, thus killing the breast stromal fibroblasts that support the growth of breast cancer. We have identified an appropriate animal model that allows for evaluation of both stromal and epithelial targeting of BT-474 xenografts. Although epithelial targeting was accomplished, stromal targeting of FAP remains under development utilizing additional antibody

reagents. These initial biodistribution studies will inform future therapeutic studies to investigate a radioimmunotherapeutic strategy for preclinical treatment of breast cancers in an animal model. If a radioimmunotherapy strategy is effective in inhibiting breast cancer growth in an animal model, it may lead to a novel therapeutic approach targeting the tumor stroma in patients with breast cancer.

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Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth

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Tumor-associated fibroblasts are functionally and phenotypically distinct from normal fibroblasts that are not in the tumor microenvironment. Fibroblast activation protein is a 95 kDa cell surface glycoprotein expressed by tumor stromal fibroblasts, and has been shown to have dipeptidyl peptidase and collagenase activity. Site-directed mutagenesis at the catalytic site of fibroblast activation protein, $Ser^{624} \rightarrow Ala^{624}$, resulted in an ~100,000-fold loss of fibroblast activation protein dipeptidyl peptidase (DPP) activity. HEK293 cells transfected with wild-type fibroblast activation protein, enzymatic mutant (S624A) fibroblast activation protein, or vector alone, were inoculated subcutaneously into immunodeficient mouse to assess the contribution of fibroblast activation protein enzymatic activity to tumor growth. Overexpression of wild-type fibroblast activation protein showed growth potentiation and enhanced tumorigenicity compared with both fibroblast activation protein S624A and vector-transfected HEK293 xenografts. HEK293 cells transfected with fibroblast activation protein S624A showed tumor growth rates and tumorigenicity potential similar only to vector-transfected HEK293. *In vivo* assessment of fibroblast activation protein DPP activity of these tumors showed enhanced enzymatic activity of wild-type fibroblast activation protein, with only baseline levels of fibroblast activation protein DPP activity in either fibroblast activation protein S624A or vector-only xenografts. These results indicate that the enzymatic activity of fibroblast activation protein is necessary for fibroblast activation protein–driven tumor growth in the HEK293 xenograft model system. This establishes the proof-of-principle that the enzymatic activity of fibroblast activation protein plays an important role in the promotion of tumor growth, and provides an attractive target for therapeutics designed to alter fibroblast activation protein–induced tumor growth by targeting its enzymatic activity.

Key Words: Fibroblast activation protein • tumor stromal fibroblasts • serine protease • tumor growth • animal model

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